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(54) Title: METHODS TO ENHANCE AND CONFINE GENE EXPRESSION IN CANCER THERAPY

(57) Abstract: The present invention provides a novel approach to gene therapy of restricted areas such as tumors. The methods introduced here comprise: (a) placing a gene of interest in a plasmid vector driven by a tissue specific promoter or a heat or light inducible promoter; and (b) modifying this vector by including a tetracycline responsive fusion protein which acts as a transcriptional activator, thus permitting regulation of gene expression by varying the levels of tetracycline; (c) modifying this vector by including DNA sequences that reduce or eliminate expression of genes in unintended cells. Also provided are a set of vectors for both sustained and regulated expression. There are also presented novel vectors for the gene therapy treatment of metastatic breast, ovarian and prostate cancer.

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METHODS TO ENHANCE AND CONFINE
GENE EXPRESSION IN CANCER THERAPY

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BACKGROUND OF THE INVENTION

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Field of the Invention

The present invention relates generally to the fields of gene therapy and cancer chemotherapy. More specifically, the present invention presents a method of controlling the expression

5   of therapeutically useful gene products via tissue specific promoters, tumor specific promoters, and inducible promoters to allow enhanced expression of these genes in tumor cells while reducing the background expression of these genes in nontarget cells.

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Description of the Related Art

One of the major obstacles to the success of cancer treatment is the difficulty in achieving tumor cell-specific killing. The inability of cytotoxic, chemotherapeutic agents to distinguish

15   between tumor and normal cells necessarily limits the dosage that can be applied. The low dosage used, which avoids killing too many normal cells, does not allow for effective killing of all tumor cells. As a result, disease relapse due to residual surviving tumor cells is frequently observed.

20   Attempts have been made to overcome these problems. These include such strategies as modifying the schedule of cytotoxic drug administration and using cytokines to counter the toxicity of chemotherapy. Another strategy, which has gained in popularity in recent years, is to produce cytotoxic

25   agents *in situ* at the tumor site. For example, attempts have been made to deliver the TNFα gene to tumor cells via adenoviral vectors and/or liposomes. However, as the expression of the TNFα gene is not restricted to the tumor sites due to the leakiness

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of the promoter, this strategy suffers from the same problem of toxicity as the systemic delivery of the cytokine itself.

In an attempt to overcome this problem, Weichselbaum and colleagues made use of the radiation inducible promoter of the early response gene (Egr-1) to activate expression of the TNFα gene *in situ*. Combined treatment with this plasmid and radiation resulted in the partial regression of a xenografted tumor. However, as the activity of the Egr-1 promoter is transient, the level of TNFα dropped off precipitously within 24 hours. Further decreases in serum levels of TNFα coincided with regrowth of the tumors (1,2). Furthermore, the high basal promoter activity of the Egr-1 promoter (which is the same as the serum response element) conferred extremely high levels of TNFα in normal cells unintended as targets. Likewise, the heat inducible HSP promoter, which has been used to direct the expression of the genes such as cytokine IL-2 (3) suffers from the same problems of transient expression, weak promoter activity and high (>25%) background activity in uninduced cells. Tissue specific promoters, while of promise, have not been shown to overcome the problems of the leakiness and the intrinsic weakness of the promoters.

In summary, results from these and other studies point to several problems with these promoter systems which render them useless for therapeutic purposes. One problem involves the leakiness of the promoters. While tissue specific promoters are suppose to restrict gene expression to specific tissues only, background expression in other tissue types is frequently the norm. For example, the PSA promoter, in addition

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to directing expression in the prostate, stimulates the expression of genes in other tissues as well. The problem of background expression with the heat or light inducible promoters is worse as both HSPp and EGRp stimulate more than 25% of the inducible

5   expression in the absence of induction. While alpha-fetoprotein promoter has been used direct toxic gene expression in hepatoma cells, the promoter also causes expression of the toxic gene in normal liver cells. As it has not been possible to deliver genes only to tumor cells *in vivo*, the leakiness of these promoters leads

10   to the unintended *in situ* expression of cytotoxic agents in normal cells.

Another major problem which limits the amount of cytotoxic agents produced is the weakness of the promoters. In order for a cytotoxic agent to kill 100% of the tumor cells, its

15   concentration at the tumor site must be equal to or greater than the concentration needed to kill all the tumor cells *in vitro* (cell culture). A weak promoter will not be able to drive expression of sufficient levels of the cytotoxic agents. The problem is compounded by the fact that not every tumor cell will take up

20   the DNA plasmid, and thus, the amount of cytotoxic agents that can be produced at the tumor site will be further limited. While it has been suggested that repeated administration may help to improve the treatment outcome, it is not clear if repeated delivery of a gene vector which expresses a suboptimal low

25   dosage of cytotoxic agent will be useful, not withstanding the problem of immune response which constraints the number of repeated treatments. While it may be conceivable to deliver an overwhelmingly large dosage of plasmids, the problem of the

leakiness of the promoter has prevented such an approach. The substantial basal activity (20-30%) has limited the administration of the heat and light inducible plasmids to small dose intra-tumor injections to minimize systemic toxicity.

5   In summary, if a promoter system is to become generally applicable for expressing cytotoxic genes *in situ* for cancer treatment, it is necessary to eliminate or greatly reduce its background activity in normal cells. More importantly, a way must be found to ensure a sufficient level of expression of therapeutic genes placed under the control of the promoter. Ideally, this level is equal to or greater than the minimal level that kills all tumor cells *in vitro*. Finally, current methods of gene therapy do not allow the delivery of genes to 100% of the intended target cells. A way also must be found to overcome this shortcoming so that even if only a portion of the target cells receive and express the therapeutic gene, most or all of the intended target cells are exposed to the therapeutic effect of the gene product.

The prior art is deficient in the lack of an effective means of preventing unwanted toxic side effects from chemotherapy and gene therapy treatments of cancer. In particular, the prior art is deficient in means of enhancing and sustaining gene expression in the intended cells at a level sufficient for killing most or all of the tumors cells at the tumor site while minimizing the side effects on normal cells. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The current invention provides compositions and methods for the controlled activation of expression of DNA molecules encoding therapeutic agents for gene therapy. Activation of expression of these DNA molecules leads to the production of protein products which may then provide opportunities for therapeutic manipulation of cells containing said DNA molecules. This may be achieved via alterations in cell growth and metabolism of the targeted cells and may include effects on neighboring cells via the secretion of therapeutic products or the conversion of non-toxic prodrugs into diffusible cytotoxic drugs. The invention offers the options of sustained activation or activation regulated by the application of antibiotics (tetracycline or its derivatives). The invention further provides novel expression vectors for use in gene therapy of local and metastatic breast, ovarian, prostate cancer and other cancers for which tissue specific promoters are available.

An original strategy to confine and enhance therapeutic gene expression to tumors spatially and temporally is also presented, in the form of novel expression vectors designed for use in local and metastatic breast, ovarian, prostate and other cancers.

The current invention is directed to the recombinant expression of cytotoxic genes such as cytokines, genes encoding prodrug-converting enzyme under the control of a tetracycline promoter. This promoter is activated by the binding of an

enhancer-promoter-binding fusion protein in the presence of a specified amount of tetracycline or its derivatives.

The instant invention is directed to a recombinant vector containing an isolated nucleic acid molecule including, but not limited to, molecules disclosed above which can be used for the sustained regulated expression of a gene under the control of a tissue specific promoter or tumor specific promoter. Such promoters include the probasin promoter, the c-erbB2 promoter, the PSA promoter, and other tissue specific promoters. Recombinant vector, pTF-0 (Tissue specific, feed forward vector), is used for the purpose of enhancing the expression in tumor cells while reducing background levels of expression of cytotoxic genes, comprising the following five cassettes:

Cassette No. 1: TSP is the tissue specific promoter and ptet is the tet operator consisting of the 19 bp inverted repeats of the operator O2 of TN10 (5) to which the tet repressor and Tet-On bind; Tet-On is a fusion of the coding sequences for amino acids 1-207 of the tetracycline (tet) repressor and the C-terminus last 130 amino acid transcription activation domain of the VP16 protein of the herpes simplex virus.

Cassette No 2: the cytotoxic gene is either a single therapeutic gene or two or more therapeutic genes each placed under the control of the tetracycline promoter (tetp). Examples of TSP include the probasin promoter for prostate cancer and the cerbB2 promoter, the whey acidic protein promoter (WAPp), or the stromelysin 3 promoter (ST3p) for breast and ovarian cancer, and other tissue specific promoters. The c-erbB2 promoter also can be used in prostate cancer.

Cassette No. 3: a gene cassette comprised of either a heat or light inducible promoter H/L-p driven Tet-On. For the heat inducible promoter, the heat shock promoter HSPp consisting of the heat shock response element (-260 to 30) of the human heat shock 70 gene promoter (6) linked to the minimal CMV promoter, pCMV (4). For the light inducible promoter Egr-1p the radiation-inducible promoter consisting of fragment -425 to +65 of the Egr-1 promoter containing four copies of the CArG domain is used.

Cassette No. 4: A cassette consisting of the tetracycline transcription repressor tetR which binds to and represses transcription from the tetracycline promoter in the absence of tetracycline or its derivatives to prevent non-specific transcription from this promoter.

Cassette No. 5: A cassette consisting of a dominant negative transcription repressor DN-Tet-On which binds to and represses transcription from the tetracycline promoter in the presence of tetracycline or its derivatives to compete with the transcription activator Tet-On. The dominant negative DN-Tet-On consisting of the coding sequences for amino acids 1-207 of the Tet repressor placed under the control of the minimal pCMV promoter.

The present invention further comprises a method in an alternate cycle of addition and removal of tetracycline is implemented such that the background amplified Tet-On is allowed to decay back to the basal level at the beginning of the addition of tetracycline. While the Tet-On amplified in the tumor cells will also decay, an excess of Tet-On remains even as

the level of Tet-On in the background has returned to the basal level. Thus, in the following cycle of addition of tetracycline, the feed forward reaction in the tumor cells starts from an even higher level of Tet-On whereas that in the background starts with

5   the same level as in the previous cycle. As such, the difference in the Tet-On and the cytotoxic drug as well increase with succeeding cycles of addition and removal of tetracycline.

It is specifically contemplated that pharmaceutical compositions of the present invention may be prepared for the

10   purpose of gene therapy. In such a case, the composition comprises the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in the art of cancer chemotherapy would readily be able to determine, without undue experimentation, appropriate dosages and routes

15   of administration. For gene therapy, the gene of interest contained in one of the plasmid vectors of the present invention, could be delivered to the target cell via a viral vector or liposome.

Another embodiment of the present invention, there is provided a general method for sustained and enhanced

20   expression of a gene via the activity of a tissue specific promoter and a tetracycline inducible promoter acting on a fusion protein which binds to a tetracycline promoter element.

Other and further aspects, features, and advantages of the present invention will be apparent from the following

25   description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

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BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features,
5   advantages and objects of the invention, as well as others which
will become clear, are attained and can be understood in detail,
more particular descriptions of the invention briefly summarized
above may be had by reference to certain embodiments thereof
which are illustrated in the appended drawings.   These drawings
10   form a part of the specification.   It is to be noted, however, that
the appended drawings illustrate preferred embodiments of the
invention and therefore are not to be considered limiting in their
scope.

Figure 1 shows a schematic representation of the
15   plasmid, pTF-0 (Tissue specific, feed forward promoter plasmid),
which consists of five cassettes as follows: In cassette 1, Tet-On is
a fusion of the coding sequences for amino acids 1-207 of the
tetracycline (tet) repressor and the C-terminal 130 amino acid
transcription activation domain of the VP16 protein of the herpes
20   simplex virus (4).   The Tet-On sequence is placed under the
control of the TSP (c-erbB-2 promoter is shown) and the tet
operator binding site and minimal pCMV.   TSP is the tissue
specific promoter, which can be the probasin promoter, c-erbB-2
promoter, and any other tissue specific promoters.   In cassette 2,
25   four copies each of the therapeutic cytotoxic genes are each
placed under the control of the tetp-pCMV promoter.   tetp is the
tet operator consisting of the 19 bp inverted repeats of the
operator O2 of TN10 (5) to which the tet repressor and Tet-On

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bind.   (In this example, the cytotoxic gene encoding interferon gamma IFN-gamma, the cytotoxic gene encoding tumor necrosis factor TNF-alpha, and the prodrug converting enzyme gene cytosine deaminase are shown.)   In cassette 3, the Tet-On sequence is placed under the control of the heat or light inducible promoter H/L-p (the heat inducible promoter HSPp is shown).   In cassette 4, the tetracycline transcription repressor tetR is placed under the control of the CMV promoter. In cassette 5, a dominant negative DN-Tet-On consisting of the coding sequences for amino acids 1-207 of the Tet repressor is placed under the control of the minimal pCMV promoter.

Figure 2 shows the expression kinetics of p53 in the H358 lung carcinoma cell line by the feed-forward reaction, where a, b, c, d, and e represent the levels of p53 reached at 10 hours after the feed-forward reaction.   Six hours after heat shock, transfected cells were treated with different doses of doxycycline. At various time points after the addition of doxycycline, the cells were stained with a p53 antibody. For each point, digital images of fifty immunostained cells were captured using a Nikon microscope.   The amount of protein expressed in each cell is proportional to the intensity of staining which was expressed as $I = 1/T$ where T is a measure of the transmitted light/unit area. This plot shows the results of one such experiment at 0.01-0.1 µg/ml of doxycycline.

Figure 3 depicts the ability of a CMVp driven dominant negative Tet-On (DN-Tet-On) to compete with the Tet-On transactivator for the tetp binding sequence and thereby attenuating the feed forward reaction.   Tet-On and DN-TET-On

were cotransfected with the ptet-p53 plasmid and the amount of p53 synthesized detected with Pab421 antibodies.

Figure 4 depicts the hypothetical feed forward kinetics of Tet-On in LnCap cells using pTF-0 in which the TSP is the probasin promoter versus the same promoter in the non-prostate cell Saos-2 under the situation when cells are not heated or irradiated.

Figure 5 depicts the hypothetical feed forward kinetics of Tet-On in LnCap cells using pTF- 0 in which the TSP is the probasin promoter versus the same promoter in the non-prostate cell Saos-2 under the situation when cells are heated or irradiated when the HSP promoter or the Egr-p promoter were used respectively.

Figure 6 depicts the theoretical feed forward reaction in the tumor cells (TL) versus the unintended target normal cells (BL).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J.

Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)];

5   B. Perbal, "A Practical Guide To Molecular Cloning" (1984). Therefore, if appearing herein, the following terms shall have the definitions set out below.

As used herein, the term "heat" is used to mean heat energy generated by any means, including microwaves.

10   As used herein, the term "light" is used to mean light energy with frequencies in the visible as well as the invisible spectrum, including ionizing radiation generated by any means. This would include a radiation source such as radionuclides capable of emitting gamma and or beta particles, or by a linear

15   accelerator. As used herein, the term "cDNA" shall refer to the DNA copy of the mRNA transcript of a gene.

As used herein, the term "derived amino acid sequence" shall mean the amino acid sequence determined by reading the triplet sequence of nucleotide bases in the cDNA.

20   As used herein the term "screening a library" shall refer to the process of using a labeled probe to check whether, under the appropriate conditions, there is a sequence complementary to the probe present in a particular DNA library. In addition, "screening a library" could be performed by PCR.

25   As used herein, the term "PCR" refers to the polymerase chain reaction that is the subject of U.S. Patent Nos. 4,683,195 and 4,683,202 to Mullis, as well as other improvements now known in the art.

The amino acids described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is

5   retained by the polypeptide. NH2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243:3552-59 (1969), abbreviations

10   for amino acid residues are known in the art.

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted

15   that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA

20   replication *in vivo*; i.e., capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

25   A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the

molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.   In discussing the structure herein

5   according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

10   A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences.   The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a

15   translation stop codon at the 3' (carboxyl) terminus.   A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences.   A polyadenylation signal and transcription

20   termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide

25   for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence.

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For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate

5   transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters often, but not always, contain "TATA" boxes, "CAAT"

10   boxes, and other regulatory sequences. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of

15   another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

20   A "signal sequence" can be included near the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell

25   before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

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The term "oligonucleotide", as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact

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sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer

5   sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementary with the sequence or hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the terms "restriction endonucleases"

10 and "restriction enzymes" refer to enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated

15 (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a

20 chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived

25 from a single cell or ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90% or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

A "heterologous' region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine,

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Texas Red, AMCA blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

Proteins can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re.

Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase, β-glucuronidase, β-D-glucosidase, β-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Patent Nos. 3,654,090, 3,850,752, and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

As used herein, the term "host" is meant to include not only prokaryotes but also eukaryotes such as yeast, plant and animal cells. A recombinant DNA molecule or gene which encodes a protein of the present invention can be used to transform a host using any of the techniques commonly known to those of ordinary skill in the art. Prokaryotic hosts may include *E. coli*, *S. tymphimurium*, *Serratia marcescens* and *Bacillus subtilis*.

Eukaryotic hosts include yeasts such as *Pichia pastoris*, mammalian cells and insect cells.

In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the
5   inserted DNA fragment are used in connection with the host. The expression vector typically contains an origin of replication, promoter(s), terminator(s), as well as specific genes which are capable of providing phenotypic selection in transformed cells. The transformed hosts can be fermented and cultured according
10   to means known in the art to achieve optimal cell growth.

The present invention is directed towards new methods of gene therapy and chemotherapy for confined areas such as tumors. In accordance with the above-mentioned object, there is provided a novel mechanism for regulating gene
15   expression via plasmids containing elements which are tissue specific and/or elements which are heat or light activated in combination with elements which are responsive to presence and concentration of antibiotic (tetracycline and its derivatives). In regulating gene expression, the tissue specific promoters allow for
20   greater expression levels in tumor cells versus normal cells of a fusion gene product Tet-On. Tet-On is a transactivator that binds to the tetp element. Amplification of Tet-On and cytotoxic genes under the control of tetp will initiate only in the presence of the antibiotic (tetracycline and its derivatives). In the presence of
25   tetracycline or its derivatives, Tet-On is activated and binds to the tetp element, activating the expression of more Tet-On and the cytotoxic gene under the control of a tetp. Concentration of the antibiotic controls the level and duration of the gene

expression. This feed forward reaction allows an ever increasing level of cytotoxic drugs or prodrug converting enzymes to be synthesized which is higher than the level of expression driven by the tissue specific promoter alone. The feed forward reaction ensures that the therapeutic level of a cytotoxic agent is eventually reached. As the rate of feed forward reaction by Tet-On is dependent on its initial concentration, the greater excess of the initial concentration of Tet-On in the cancer cells of the tissue of origin in which the tissue specific promoter is active compared to cells of other tissue types allows a faster feed forward rate. As a result, by the time the therapeutic level is reached in the tumor cells, the background level amplified in the normal cells still remains at a lower level. This contrasts with conventional chemotherapy in which all cells (tumor and normal cells) are exposed to the same concentration of chemotherapeutic drugs.

In the case of where the difference in the promoter activity between the tumor cells and the normal cells is large, the feed forward rate, which is dependent on the initial concentration of Tet-On, will also be large and will increase with the feed forward reaction. The level of cytotoxic drug in the background will not have reached an unacceptable toxic level when the tumor site reaches the therapeutic level. However, in the case where the difference in the promoter activity in the tumor cells and the normal cells is insufficiently large, the level of the background may be at a unacceptable level when the therapeutic level is reached at the tumor site. Therefore, the success of this gene therapy/chemotherapy approach relies on enhancing the difference in the level of expression of cytotoxic

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agents in the tumor cells and the normal cells. The present invention provides three mechanisms for the enhancement of gene expression in the tumor cells and suppression of gene expression in unintended cells for use in those cases where the difference in the promoter activity in the tumor cells and the normal cells is insufficiently large.

In the first method, an alternate cycle of addition and removal of tetracycline is implemented such that the background amplified Tet-On is allowed to decay back to the basal level at the beginning of the addition of tetracycline. While the Tet-On amplified in the tumor cells will also decay, there will remain an excess of Tet-On even as the level of Tet-On in the background has returned to the basal level. Thus, in the following cycle of addition of tetracycline, the feed forward reaction in the tumor cells starts from an even higher level of Tet-On whereas that in the background starts with the same level as in the previous cycle. As such, the difference in the Tet-On and the cytotoxic drug as well increase with succeeding cycles of addition and removal of tetracycline.

In a second method, a CMV promoter driven dominant negative Tet-On gene cassette is incorporated into the therapeutic plasmid. Because the dominant negative Tet-On is driven by the CMV promoter, a fixed amount of which will be produced. The dominant negative Tet-On competes to attenuate an amount of feed forward reaction in proportion to the amount of dominant negative Tet-On produced. As such, when an equal amount of Tet-On mRNA is blocked and an equal amount of feed forward reaction is attenuated in both the tumor and the

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background cells, the difference in the amount of Tet-On and the rate of the feed forward reaction in the tumor cells and the background increases.

In a third method, one or more heat or light inducible promoter driven Tet-On is incorporated in the therapeutic cassette with the dominant negative Tet-On. Although heat or light inducible promoters have a high background activity in all cells, expression of the Tet-On and the therapeutic gene due to the background activity of the heat or light inducible promoters is suppressed by the constitutive expression of the antisense and dominant negative DNA in the same plasmid. When the tumor cells are heated or irradiated, a transient increase of 3 to 4-folds of Tet-On is induced. As such, the irradiated tumor cells at the tumor site initiate the feed forward reaction in the presence of tetracycline at a much greater rate than the unheated, un-irradiated normal cells.

In the fourth method, a CMV promoter-driven tetracycline repressor tetR is included in the plasmid. In the absence of tetracycline and its derivatives, this tetR binds to the tetp promoters and prevent non-specific transcription of Tet-On and the cytotoxic genes. In the presence of tetracycline and its derivatives, the tetR cannot bind to the tetp and the transcription is then regulated by the enhancer-promoter-binding fusion protein Tet-On and DN-Tet-On which then bind to the tetp.

In one aspect of the present invention, there is provided a recombinant vector containing an isolated nucleic acid molecule, which can be used for the expression of cytotoxic genes such as cytokines, genes encoding prodrug-converting

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enzyme, under the control of a tetracycline promoter. This promoter is activated by the binding of an enhancer-promoter-binding fusion protein in the presence of a specified amount of tetracycline or its derivatives.

5   In yet another aspect of the present invention, there is provided a recombinant vector containing an isolated nucleic acid molecule including, but not limited to, molecules disclosed above which can be used for the sustained regulated expression of a gene under the control of a tissue specific promoter or tumor

10   specific promoter. Such promoter includes the probasin promoter, the c-erbB2 promoter, the PSA promoter, and other tissue specific promoters.

          In another aspect of the present invention, there is provided a recombinant vector, pTF-0 (Tissue specific, feed

15   forward vector), for the purpose of enhancing the expression in tumor cells while reducing background levels of expression of cytotoxic genes, comprising the following five cassettes: In cassette (1), TSP is the tissue specific promoter and ptet is the tet operator consisting of the 19 bp inverted repeats of the operator

20   O2 of TN10 (5) to which the tet repressor and Tet-On bind; Tet-On is a fusion of the coding sequences for amino acids 1-207 of the tetracycline (tet) repressor and the C-terminus last 130 amino acid transcription activation domain of the VP16 protein of the herpes simplex virus; In cassette (2), a cytotoxic gene is either a

25   single therapeutic gene or two therapeutic genes linked by an IRES placed under the control of the tetracycline promoter (tetp). Examples of TSP include probasin promoter for prostate cancer and cerbB2 promoter, whey acidic protein promoter (WAPp), or

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stromelysin 3 promoter (ST3p) for breast and ovarian cancer, and other tissue specific promoters. The c-erbB2 promoter also can be used in prostate cancer. In cassette (3) a gene cassette comprised of a HSPp driven Tet-On. HSP is the heat shock

5   promoter consisting of the heat shock response element (-260 to 30) of the human heat shock 70 gene promoter (6) linked to the minimal CMV promoter, pCMV (4). In a different version of pTF-0, the HSPp is replaced with the radiation inducible promoter Egr-1p which is the radiation-inducible promoter consisting of

10 fragment -425 to +65 of the Egr-1 promoter containing four copies of the CArG domain. In cassette (4) the tetracycline transcription repressor tetR which is placed under the control of the minimal pCMV promoter. In cassette (5), a dominant negative DN-Tet-On consisting of the coding sequences for amino

15 acids 1-207 of the Tet repressor is placed under the control of the minimal pCMV promoter.

The present invention is also directed to a method in which sustained expression of a gene or set of genes under control of a heat or light inducible promoter is achieved by

20 exposure to heat or light comprising the step of introducing the vector containing said gene(s) into the host organism and applying heat or light energy.

It is specifically contemplated that pharmaceutical compositions of the present invention may be prepared for the

25 purpose of gene therapy. In such a case, the composition comprises the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in the art of cancer chemotherapy would readily be able to determine,

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without undue experimentation, appropriate dosages and routes of administration. For gene therapy, the gene of interest contained in one of the plasmid vectors of the present invention, could be delivered to the target cell via a viral vector or liposome.

5   The level of ordinary skill of the average scientist in the area of molecular cancer biology has increased substantially in recent years. A person having ordinary skill in this art would readily be able to construct and utilize the plasmids for this novel approach to gene therapy given the teachings of the present

10   specification.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

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EXAMPLE 1

The pTF-0 vector: structure and mode of action

Figure 1 is a schematic depiction of the pTF-0 vector.

20   The pTF-0 plasmid functions via a feed-forward reaction to amplify the expression of Tet-On and a cytotoxic gene. In the tumor cells, the tissue specific promoter directs the synthesis of a large amount of the Tet-On product. In the background normal cells that take up pTF-0 background expression, the leakiness of

25   the tissue specific promoter results in the synthesis of a comparatively lower level of Tet-On. In the absence of tetracycline, the tetR repressor binds to the tetp and prevents nonspecific transcription of the Tet-On and the cytotoxic genes.

In the presence of tetracycline, the Tet-On protein becomes active and the feed-forward reactions proceed at different rates in the tumor cells versus the background cells. This reaction is controlled by the level of tetracycline. As such, cytotoxic drug expression is elevated until tetracycline is removed. When the cytotoxic agent in the tumor cells has reached a therapeutic level, the amount of the drug in the background would still be low enough as to not cause an unacceptable level of systemic toxicity.

EXAMPLE 2

Verification of the concept of amplifiable and sustained expression of Tet-On and p53 with the feed forward inducible promoter

To further validate the concept of tetracycline feed-forward amplification of gene expression and the utility of the HSP promoter in conjunction with the tetp promoter, two plasmids were constructed. The plasmid ptet-splice p53wt was constructed by subcloning a wild-type p53 cDNA into the ptet-splice vector (Gibco BRL) which places p53 under the control of the tetp promoter (which consists of the regulatory sequences from the tetracycline-resistance operon upstream of a minimal mCMVp promoter). The plasmid HSP-tetp-Tet-On was constructed by replacing the CMV promoter in pTet-On (Clontech) with 300 bp of the human heat shock protein promoter and the tetp promoter.

H358, a non-small cell lung carcinoma cell line with a homozygous deletion of p53, was grown in RPMI + 10% fetal calf serum.   Ten   million   exponentially   growing   cells   were cotransfected with 50 μg of "ptet-splice p53wt" and 10 μg of

5   "HSP-tetp-Tet-On" by electroporation using a BRL cell-Porator at 1180 μF and 240 V in 0.8 ml RPMI + 6 mM glucose. Transfected cells were plated out at 25% confluence for 36 hours and then half of them were heat-shocked at 45°C for twenty minutes.   Six hours after heat shock, cells were treated with different doses of

10 doxycycline.   At   various   time   points   after   the   addition   of doxycycline, the cells were stained   immunohistochemically with the monoclonal p53 antibody DO-1 (Santa Cruz Biologicals) using an   immunoperoxidase   cell   staining   kit   (Vector)   and diaminobenzidine (DAB).   For each point, the digital images of

15 fifty   immunostained   cells   were   captured   using   a   Nikon microscope.   The amount of protein expressed in each cell is proportional to the intensity of staining which was expressed as I = 1/T   where T is a measure of the transmitted light/unit area. Results of one such experiment at 0.01-0.1 μg/ml of doxycycline

20 are shown in Figure 2.

When 0.1 μg/ml of doxycycline was added at 6 hours after heating (when the level of induced Tet-On should have been at its peak), more than 12 fold amplification of p53 was reached in 10 hours (curves a and b, Figure 2).   During this time,

25 doxycycline also started a feed-forward reaction in the unheated cells as indicated by the substantial level of Tet-On.   However, since the amplification started off from a lower level, the

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amplified level of Tet-On at 10 hours reached only a low level (curves c and d, Figure 2).

5   It is possible to regulate the level of induced p53 in the feed-forward system with an alternate regimen of tetracycline addition and removal. In the time it takes for Tet-On (e.g. Figure 2 level [c]) in the unheated cells to decline back to background level [e] after removal, the level of Tet-On in the heated cells [a] would have declined by a similar proportion (which is equal to [c]-[e]). However, since this level([a]-[c]-[e]) is much higher than in the unheated cells [e], the addition of tetracycline will re-start the feed-forward reaction for the heated cells from a much higher level ([a]-([c]-[e])). As such, the level of background p53 in unheated cells can be kept at or below the low level reached at 10 hours ([c]), whereas the p53 level in heated cells will continue to escalate. Thus, while the TNFα and p53 driven by the HSP promoter directly is transient, the expression driven by the feed-forward system is on for as long as tetracycline is available. Since the regimen of tetracycline addition *in vivo* will be determined by the decay rate of tetracycline *in vivo*, it is important to know the half-life of the Tet-On in tumor cells.

EXAMPLE 3

DN-Tet-On attenuation of the feed forward reaction

25   The pTF-0 plasmid functions by a feed forward reaction to amplify the expression of Tet-On and the cytotoxic agent. As was shown in example 2, in the presence of tetracycline, the background can be amplified to a great extent

given enough time. However, this feed forward reaction may be prevented by the expression of the dominant negative Tet-On protein which competes for the same DNA binding site on the pTet promoter. While the amplification in the tumor cells will also be affected by the same magnitude, the greater initial amount of Tet-On will allow the feed forward to proceed.

To demonstrate that a dominant negative Tet-On can compete to suppress the amplification reaction, a plasmid containing a CMVp driven Tet-On was cotransfected with a similarly driven dominant negative Tet-On. p53 is driven by a tetracycline promoter. pTet-On, DN-pTet-On and pTet-p53 were cotransfected into the p53 null cell Saos2 and the level of expression of p53 in the transfectant in the presence or absence of tetracycline was detected in cell lysate with the anti-p53 antibody Pab421. The data in Figure 3 show that the DN-Tet-On worked very well in competing for the DNA binding site of the tetp promoter. These data therefore suggest that it is possible to use a DN-Tet-On to attenuate the feed forward amplification.

EXAMPLE 4

Reduction in background levels of expression

In vivo, the pharmacokinetics of tetracycline is heterogeneous for different tissues. Preferential concentration of tetracycline in specific tissues will lead to higher background expression of Tet-On in some tissues. For example, in humans, 10-35% of oxytetracycline is removed via the kidney, a

substantial amount of which is excreted in the active form. Therefore it is desirable to minimize the background expression levels at the onset to prevent run away amplification in the unintended tissues.

5   In the case of tissue specific promoters or tumor site specific promoters such as the HSP promoter in example 2, the difference in the amplified level of cytotoxic gene products in the tumor cells and the background is insufficiently large and toxicity could become a problem if the feed forward reaction is

10   allowed to proceed further.   This problem of background expression is overcome with the dominant negative Tet-On cassette placed under the control of the pCMV promoter.   The constitutively produced dominant negative Tet-On, which contains the DNA binding site for tetp but not the transcription

15   activation domain, competes for the tetp binding site.   As the feed forward reaction at any time is dependent on the concentration of the Tet-On at the start of the reaction, the increased difference in the absolute level of Tet-On between the tumor and the background cells ensures a greater rate of feed

20   forward in the tumor cells.   The DN-Tet-On works by reducing a fixed amount of feed forward reaction in proportion to the amount of DN-Tet-On synthesized.

EXAMPLE 5

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Further enhancement of the level of expression at the tumor site

          As mentioned *supra*, genes placed under the control of such promoters as the radiation-inducible promoter of the Egr-

1 gene or the heat shock promoter can be induced 3 to 4 times

above their background when cells harboring said gene are

irradiated or heated.   In non-irradiated cells, the activity of the

background Tet-On due to the leak-through transcription of Tet-

5 On by the HSP or EGRp promoter is attenuated by the DN-Tet-On.

Upon irradiation, Tet-On is transiently induced 3-4 fold and in

the presence of tetracycline enables the feed-forward reaction to

go at a much greater rate than the unirradiated cells.   Thus, a

combination of tissue specific promoter and heat or light

10 inducible promoter, together with dominant negative Tet-On will

change an undesirable feed forward profiles of some tissue

specific promoter such as the PSA promoter or a tumor site

promoter such as the HSP promoter in unirradiated or heated

cells (figure 4) into one that is favorable upon heating or

15 irradiation (figure 5).

EXAMPLE 6

20 <u>Modification of the feed forward kinetics to enhance the
difference in the level of cytotoxic drugs at the tumor site</u>

To further enhance the difference in the level of

cytotoxic drugs in tumor cells versus normal cells, a strategy of

alternate cycles of addition and removal of tetracycline can be

25 applied.   A hypothetical outcome is show in Figure 6.   Following

the addition of tetracycline, the level of cytotoxic drugs in the

tumor cells (TL) reaches a level higher than that in the

background cells (BL).   If tetracycline is removed at this point,

the feed forward reaction stops and Tet-On levels decay. After allowing the Tet-On to decay for several half-lives or until it is back to the same background level as at the start of the induction for the normal cells, tetracycline is again added to start the feed

5   forward reaction. As the level of Tet-On at the time of removal of tetracycline is higher in the tumor cells compared to normal cells, after the same amount of elapsed time, the amount of Tet-On leftover will still be high in the tumor cells whereas that in the background cells will have returned to the basal level. Thus, by

10   alternate cycles of addition and removal of tetracycline, the level of Tet-On and cytotoxic drug in background normal cells will be kept at the same level, whereas that in the tumor cells will be amplified more and more with each cycle and reach the therapeutic level eventually.

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The following references were cited herein:

(1) Treisman, R.H., (1986) Cell 46, 567-574

(2) Prywes, et al., Proc. Natl. Acad. Sci. USA 85, 7206-7210

(3) Sherman et al., Proc. Natl. Acad. Sci. (1990) 87(15): 5663-

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(4) Gossen et al., Science 268, 1766-1769 (1995)

(5) Gossen et al., PNAS 89, 5547-5551 (1992)

(6) Voellmy et al., Proc. Natl. Acad. Sci. USA 82, 4949-4953 (1985)

25   Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each

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individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and

5   obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations

10   on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1.   A recombinant vector, pTF-0 (Tissue specific, feed forward vector), said vector comprising the cassettes:

(a) a first cassette for the expression of Tet-On, wherein expression of said Tet-On gene is regulated by a tissue specific promoter and a tet operator;

(b) a second cassette for the expression of one or more genes of interest, wherein expression of each gene is regulated by a tetracycline inducible promoter;

(c)   a third cassette comprising Tet-On, wherein expression of said Tet-On gene is regulated by an energy inducible promoter selected from the group consisting of heat inducible promoters and light inducible promoters;

(d) a fourth cassette for expression of a tetracycline transcription repressor, tetR; and,

(e)   a fifth cassette for expression of a dominant negative Tet-On transcription repressor (DN-Tet-On).

2.   The vector of claim 1, wherein said Tet-On is a fusion protein comprising sequences from the tetracycline repressor and sequences from the transcription activation domain of VP16 protein.

3.   The vector of claim 2, wherein said sequences from the tetracycline repressor correspond to amino acids 1-207 of the tetracycline repressor.

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4.   The vector of claim 2, wherein said sequences from VP16 protein correspond to the C-terminal 130 amino acid of VP16 protein from herpes simplex virus.

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5.   The vector of claim 1, wherein said tet operator comprises 19 bp inverted repeats of operator O2 from tetracycline-resistance operon TN10 to which Tet-On and tetR bind.

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6.   The vector of claim 1, wherein said tetracycline inducible promoter is tetp, comprised of a minimal promoter in combination with 19 bp inverted repeats of operator O2 from

20   tetracycline-resistance operon TN10.

7.   The vector of claim 6, wherein said minimal promoter is a pCMV promoter

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8. The vector of claim 1, wherein said tissue specific promoter is selected from the group consisting of c-erbB2 promoter, WAP promoter, STP3 promoter and probasin promoter.

5

9. The vector of claim 1, wherein said energy inducible promoter is a heat shock promoter, hsp.

10   10. The vector of claim 9, wherein said heat shock promoter comprises nucleotides −260 to 30 of the promoter from the gene for human heat shock protein 70.

15   11. The vector of claim 1, wherein said energy inducible promoter is derived from light inducible Egr-1 promoter.

20   12. The vector of claim 11, wherein said energy inducible promoter comprises nucleotides −425 to 65 of the Egr-1 promoter containing four copies of a CArG domain.

25   13. The vector of claim 1, wherein said tetR gene is expressed by the CMV promoter.

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14. The vector of claim 1, wherein said dominant negative Tet-On (DN-Tet-On) comprises coding sequences for amino acids 1-207 of the Tet repressor, tetR.

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15. The vector of claim 1, wherein expression of said dominant negative Tet-On is regulated by a minimal pCMV promoter.

10

16. The vector of claim 1, wherein said genes of interest in said second cassette are therapeutic genes.

15   17. The vector of claim 16, wherein said therapeutic genes are effective against cancer.

18. The vector of claim 17, wherein said therapeutic

20   genes are selected from the group consisting of cytotoxic genes, genes for prodrug converting enzymes, and cytokine genes and tumor suppressor genes.

25   19. The vector of claim 18, wherein said cytotoxic genes are selected from the group consisting of tumor necrosis factor alpha (TNFα) and interferon gamma (IFN-γ).

20. The vector of claim 18, wherein said prodrug converting enzymes are selected from the group consisting of cytosine deaminase and HSV thymidine kinase.

5

21. The vector of claim 18, wherein said cytokines are selected from the group consisting of interleukins, interferons, and tumor necrosis factors.

10

22. The vector of claim 18, wherein said tumor suppressor genes are selected from the group consisting of p53 and Rb.

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23. The vector of claim 18 containing multiple copies of each therapeutic gene, wherein each copy is expressed from its own tetracycline inducible promoter.

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24. The vector of claim 23 containing four copies each of IFN-γ, TNF-α, and cytosine deaminase.

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25. A method for the treatment of cancer in a patient, said method comprising the steps of:

a) administering the vector of claim 17 to a patient; and

b) administering tetracycline to said patient prior to activate expression of Tet-On and said therapeutic genes.

5

26. The method of claim 25, wherein a tetracycline derivative is used to induce expression of Tet-On and said therapeutic genes.

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27. The method of claim 25, further comprising the step of augmenting expression of Tet-On and thus of said therapeutic gene comprising the step of exposing said cancer to an appropriate form of energy to activate expression of Tet-On

15 from said third cassette.

28. The method of claim 27, wherein said cancer is selected from the group consisting of local cancer and metastatic

20 cancer.

29. The method of claim 27, wherein said cancer is selected from the group consisting of breast cancer, ovarian

25 cancer, and prostate cancer.

30. The method of claim 25, wherein background expression of said therapeutic genes in nontarget cells is reduced by alternate cycles of tetracycline administration and withdrawal such that Tet-On expression is allowed to return to basal levels in
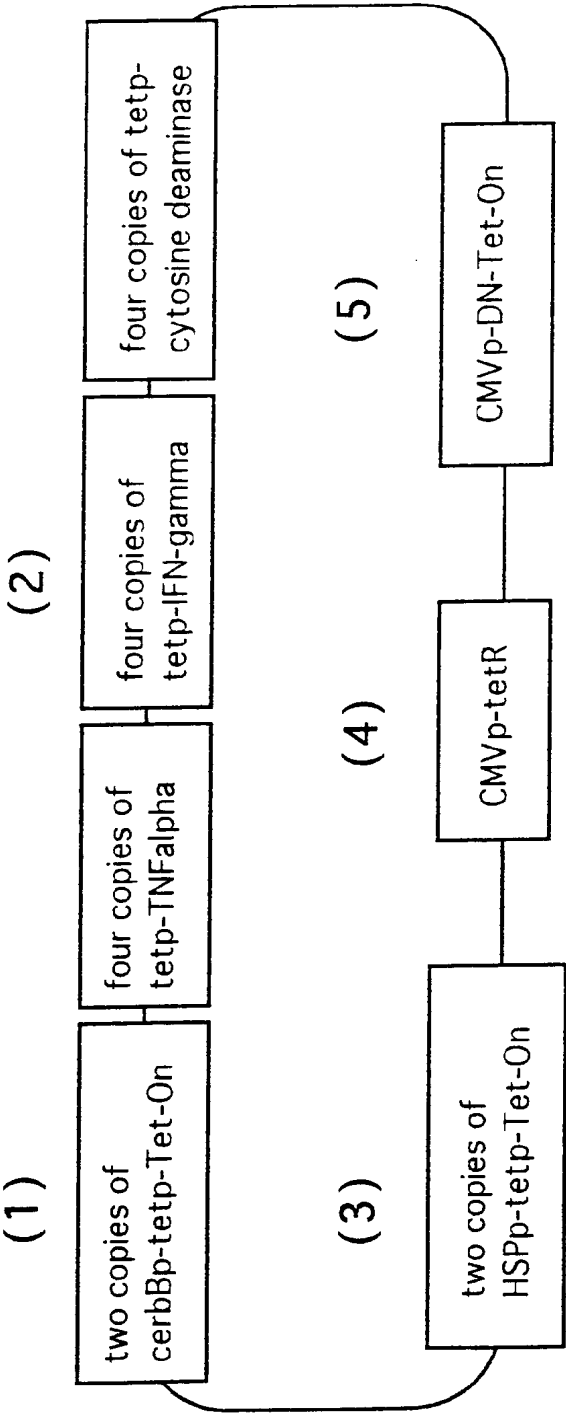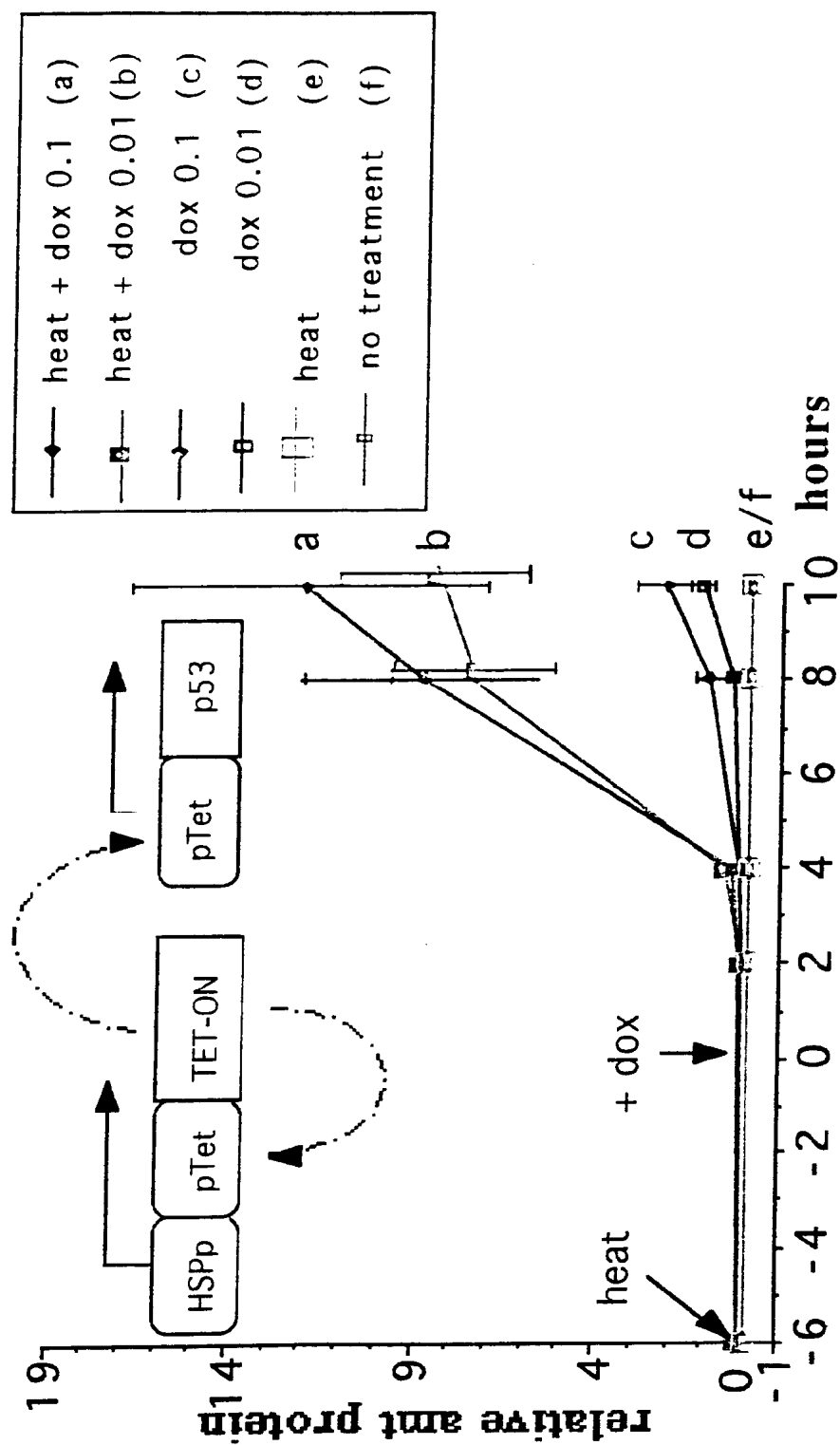5   said nontarget cells prior to each cycle of tetracycline administration.

Fig. 1

Fig. 2

pTet-p53   1 1 1 1 1 1 1 1 1 1
pTet-On   1 1 0 0 1 1 1 1 1 1
DN-pTet-On   O O 1 1 1 1 1 1 4 4
Doxycycline   - + - + - + - + - +
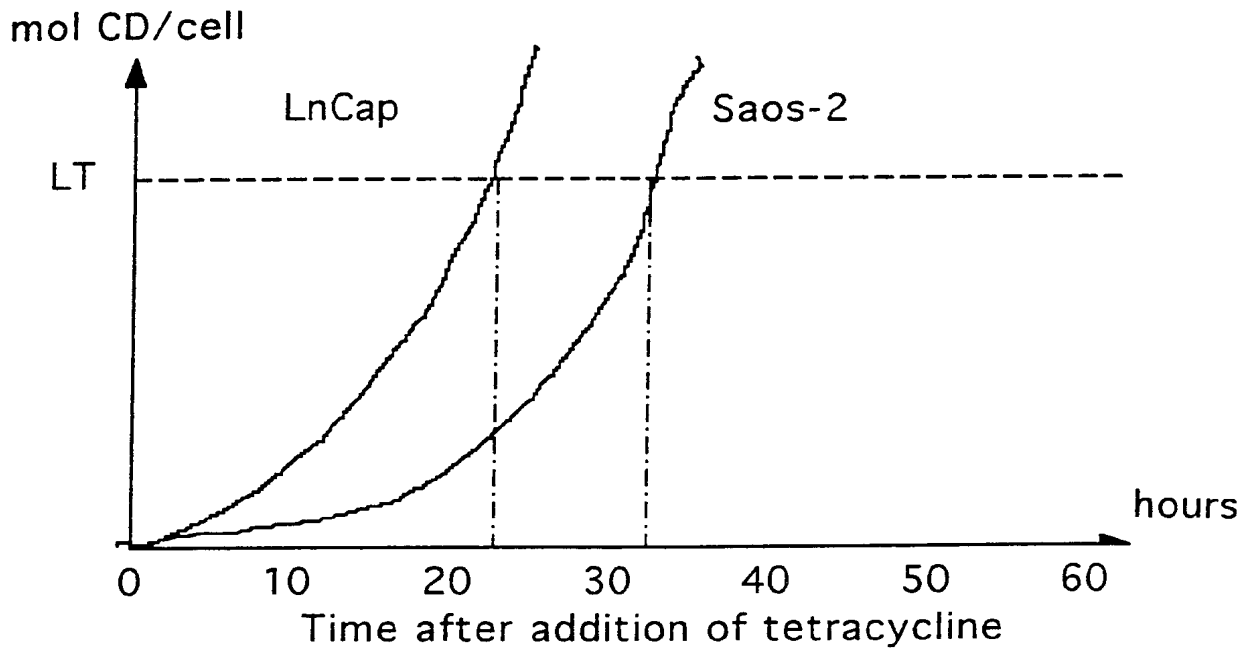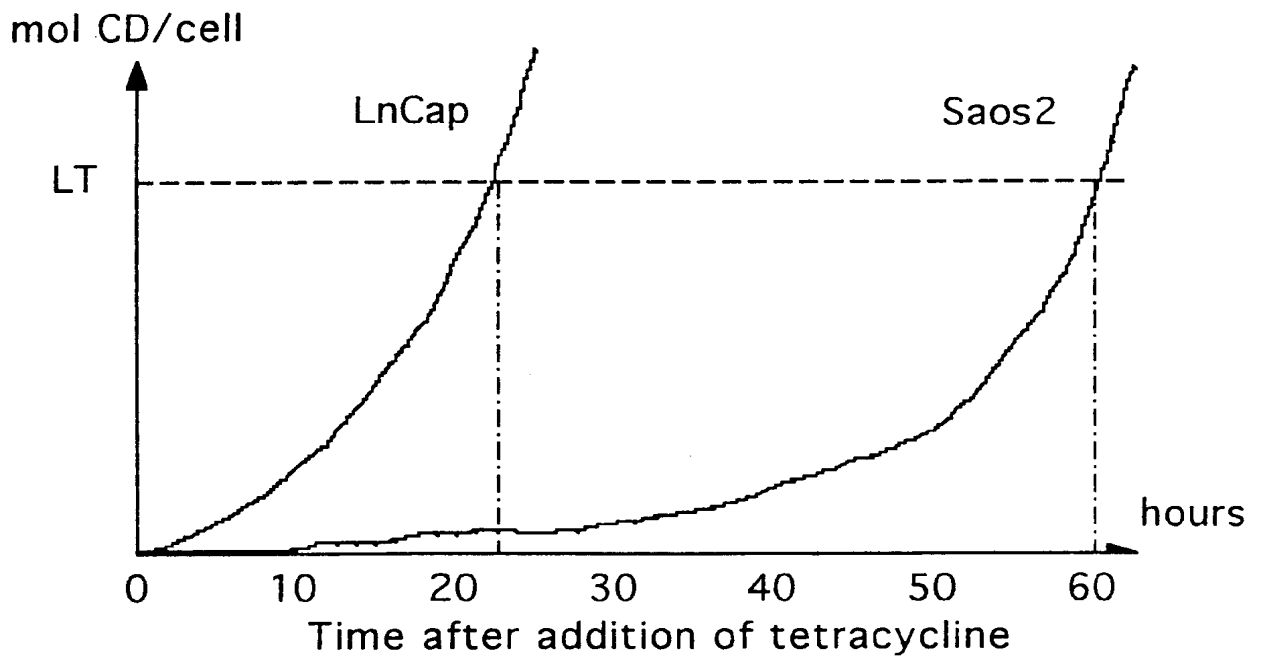p53   ►   ▬   ▬   ‑
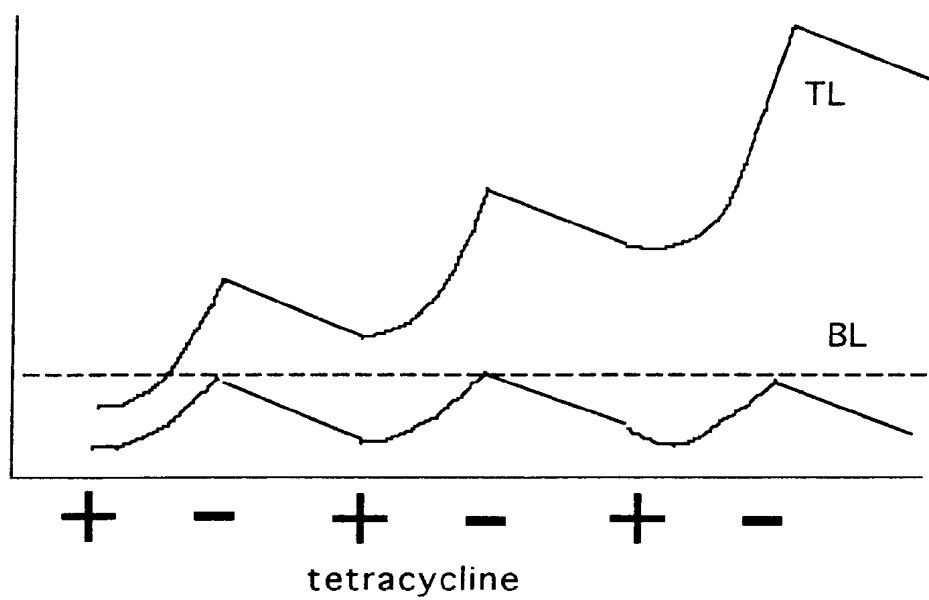
              1 2 3 4 5 6 7 8 9 10

Fig. 3

Fig. 4

Fig. 5

Fig. 6

INTERNATIONAL SEARCH REPORT	International application No.
	PCT/US00/29783

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/02, 21/04; A61K 31/70
US CL : 536/23.1, 23.5, 24.1; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.5, 24.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANDERSON, W.F. Human gene therapy. Nature. 30 April 1998, Vol. 392 (supp) pages 25-30, entire document.	1-30
Y	MOLIN, M. et al. Two novel adenovirus vector systems permitting regulated protein expression in gene transfer experiments J. Virology. October 1998, Vol. 72, No 10, pages 8358-8361, entire document.	1-30
Y	US 5,589,362 A (BUJARD et al) 31 December 1996, entire document.	1-30
Y	US 5,814,618 A (BUJARD et al) 29 September 1998, entire document.	1-30
Y	US 5,789,156 A (BUJARD et al) 04 August 1998, entire document.	1-30

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 JANUARY 2001	**29 JAN 2001**
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JOSEPH T. WOITACH
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,E	US 6,140,103 A (EINERHAND et al) 31 October 2000, entire document.	1-30
Y,P	US 5,989,910 A (MERMOD et al) 23 November 1999, entire document.	1-30